



High cut-off microdialysis catheters to clinically investigate cytokine changes following flap transfer

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Abstract

Background ‘Choke vessels’ are thought to dilate in the first 72 h when blood flow to an area is disrupted. This study used ‘high cut-off’ microdialysis catheters in clinical research to investigate factors mediating circulatory change within free flaps.

Methods Six patients undergoing DIEP flap breast reconstruction each had three ‘high cut-off’ microdialysis catheters, with a membrane modification allowing molecules as large as 100 kDa to pass, inserted into Hartrampf zones 1, 2 and 4 to assess multiple vascular territories. Microdialysis continued for 72 h post-operatively. Samples were analysed for interleukin-6 (IL-6), tumour necrosis factor alpha (TNF α) and fibroblast growth factor basic (FGF β).

Results Three hundred and twenty-four samples were analysed for IL-6, FGF β and TNF α totalling 915 analyses. IL-6 showed an increasing trend until 36 h post-operatively before remaining relatively constant. Overall, there was an increase ($p < 0.001$) over the time period from 4 to 72 h, fitting a linear trend. TNF α had a peak around 20–24 h before a gradual decrease. There was a significant linear time trend ($p = 0.029$) between 4 and 76 h, decreasing over the time period. FGF β concentrations did not appear to have any overall difference in concentration with time. The concentration however appeared to oscillate about a horizontal trend line. There were no differences between the DIEP zones in concentrations of cytokines collected.

Conclusion This study uses high-cut off microdialysis catheters to evaluate changes in cytokines, and requires further research to be undertaken to add to our knowledge of choke vessels and flap physiology.

Level of evidence: Level IV, diagnostic study.

Keywords Choke vessels · Angiosomes · Microdialysis · DIEP flaps · Flap perfusion

Introduction

Flap design is based on the concept of angiosomes or perforasomes, three-dimensional blocks of tissue supplied by a source artery and vein [1, 2]. These territories are linked to adjacent territories by reduced calibre anastomotic vessels theoretically known as ‘choke vessels’ [3]. Choke vessels are thought to dilate in the first 72 h when blood flow to an area is disrupted, with high to low pressure flow, either following surgical delay where the flap is raised in stages [4], or following immediate flap transfer. This dilation continues for 7 days

at which point the choke vessels are thought to become irreversibly larger diameter vessel. The physiological mechanism of choke vessel opening has yet to be elucidated. This clinical study was designed to use microdialysis technique in investigation of mediators that may be influential in the increase in perfusion in the first 72 h after flap transfer, using the DIEP flap as the angiosome model.

Microdialysis catheters function by using a semipermeable membrane (usually allowing molecules of up to 20 kDa to pass) and a dialysis fluid, collecting the dialysate to be analysed in a microvial. Microdialysis catheters used in this study have a modification of the semipermeable membrane allowing molecules of up to 100 kDa to be measured as has been validated in previous clinical research studies [5–7].

The purpose of the study was to use microdialysis to assess larger molecules in the tissue chemistry in relation to the period of choke vessels opening following free flap transfer, using DIEP breast reconstruction as the model. The intraoperative sites of insertion for the microdialysis catheters in this study were labelled using the original Hartrampf zones [8] 1, 2

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(contralateral midline) and 4, each zone representing an angiosome (with appreciation that zones 2 and 3 have been switched, Holm's zones [9]). Zones 1 and 4 were specifically chosen to represent contrasting quality of perfusion across the flap.

Samples were analysed for three cytokines chosen for being implicated in changes in circulation and shown in animal studies to upregulate in flaps; markers of inflammatory response tumour necrosis factor alpha (TNF α , 26 kDa) and interleukin 6 (IL-6, 22–27 kDa), and angiogenic factor fibroblast growth factor basic (FGF β or FGF2, 18 kDa).

Materials and methods

Following Ethics Committee approval (in accordance with Helsinki Declaration 1975) and informed consent of the participants, 6 patients, mean age 44.8 years (range 36–60 years) and a mean body mass index of 26.4, undergoing immediate DIEP flap breast reconstruction following mastectomy for breast cancer, participated in this study.

'High cut-off' microdialysis catheters (CMA) were used, with a dialysing membrane (10 mm length) allowing molecules up to 100 kDa to pass.

Intraoperatively, each patient had three high cut-off microdialysis catheters, a, b and c, inserted into DIEP flap zones 1, 2 and 4 respectively. Three microdialysis pumps, set at a flow rate of 0.3 μ l/min perfused isotonic 'perfusion fluid T1' (Na $^{+}$ 147 mmol/l, K $^{+}$ 4 mmol/l, Ca $^{2+}$ 2.3 mmol/l and Cl $^{-}$ 156 mmol/l) through the catheters, and the microvials collecting dialysate were changed every 4 h. The catheters were secured with an occlusive dressing.

Microdialysis continued for 76 h and vials were collected every 4 h from all three catheters and immediately stored in a -80°C freezer. Analysis was performed using enzyme-linked immunosorbent assay, ELISA (Quantikine High Sensitivity, R&D Systems) to detect human IL-6, FGF β and TNF α .

Results

The average ischaemia time during DIEP transfer and anastomosis was 93 min (range 56–135 min), and the mean flap weight 670 g (range 460–1030 g). The flaps were all based on medial perforators. Our patients had no complications related to the use of microdialysis catheters, and no patient suffered partial flap necrosis.

All six patients' samples were analysed for IL-6, FGF β and TNF α .

Patient 4 did not have zone 4 transferred with the flap and therefore only had two catheters, a and b in zones 1 and 2.

Patient 5 had no collection beyond 56 h due to the catheters dislodging inadvertently following examination for a haematoma. Patient 4 did not have a 4th zone in the flap and therefore no catheter c, and patient was not analysed for FGF β due to a technical error. Three hundred and twenty-four samples were analysed by high sensitivity ELISA for each IL-6 and TNF α , and 267 for FGF β , totalling 915 analyses from 324 vials.

The method of residual maximum likelihood (REML) was used to fit the data to a linear model, (GenStat) with fixed effects of time (T) and catheter (C), the two-factor interaction T.C, and the random effect of patient (P), with interactions P.T and P.C, with a three factor interaction P.T.C estimating the error of variance.

Interleukin-6

Figure 1 shows the mean concentration averaged over all 6 patients, and Fig. 2 the predicted mean concentration by time. There is an initial increase to 16 h, followed by relatively constant concentrations until about 52 h, then a decline. Concentrations in catheter c (zone 4) tend to be the greatest although there is no clear separation, and this is not the same at all time points.

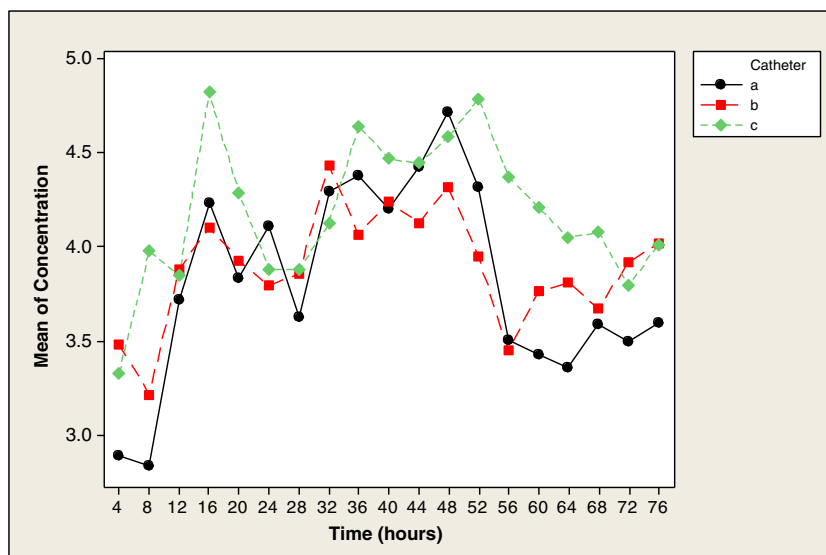
Statistical significance was assessed using approximate *F* tests of main effects and interactions. The effect of catheter is not significant ($p = 0.580$, $p = 0.592$). The effects of time, in a linear trend, are significant ($p < 0.001$). Effects of time in a non-linear trend are not significant ($p = 0.106$). The concentration of interleukin-6 (IL-6) therefore tends to increase ($p < 0.001$) over the period from 4 to 72 h, and this can best be explained by a linear trend.

Tumour necrosis factor alpha

Patients' samples were analysed as for IL-6 and FGF β (Figs. 3 and 4), the predicted concentrations by time. There do not appear to be any uniform differences between catheters and there was no obvious time trend.

The results of tests of fixed effects are shown in Table 2. The test of interaction (C.T) shows that there is no evidence that the linear trend in concentration differs between catheters ($p = 0.503$). Considering the effect of time, tumour necrosis factor alpha has a significant linear trend ($p = 0.029$, and $p = 0.028$ with catheter fitted first). There appears to be a peak concentration around 16–20 h, and overall a slightly decreasing time trend is apparent which is statistically significant. The value of the estimated trend was equal to $-0.004 \mu\text{g/ml}$ per hour.

Fig. 1 Mean concentration averaged over 6 patients versus time, by catheter, IL-6



Fibroblast growth factor basic

The five patients' samples in microdialysis vials were analysed for fibroblast growth factor basic (FGF β) (patients 1, 2, 3, 5 and 6). The vials as before were collected every 4 h from catheters a, b and c. The mean concentrations for five patients are in Fig. 5, and the predicted concentrations by time in Fig. 6. There did not appear to be any uniform differences between catheters and no obvious time trend. There did however appear to be a pattern of cyclical variation.

REML modelling of the log concentration produced a residual plot in which the residuals tended to decrease in

absolute magnitude with increasing mean concentration. The square root transformation was found to be the most appropriate in terms of homogeneity of variance and was used in this analysis (hence predicted concentrations in Fig. 6 backtransformed). The differences between catheters are not statistically significant ($p = 0.719$, and $p = 0.698$ allowing for the effect of time). Considering the effect of time, the linear trend is not statistically significant ($p = 0.530$ and $p = 0.535$ allowing for the effect of the catheter). However, the non-linear effects of time are on the borderline of statistical significance ($p = 0.050$ not allowing for catheter, $p = 0.049$ allowing for catheter). There does appear to be a cyclical variation in

Fig. 2 Predicted concentration by time, IL-6

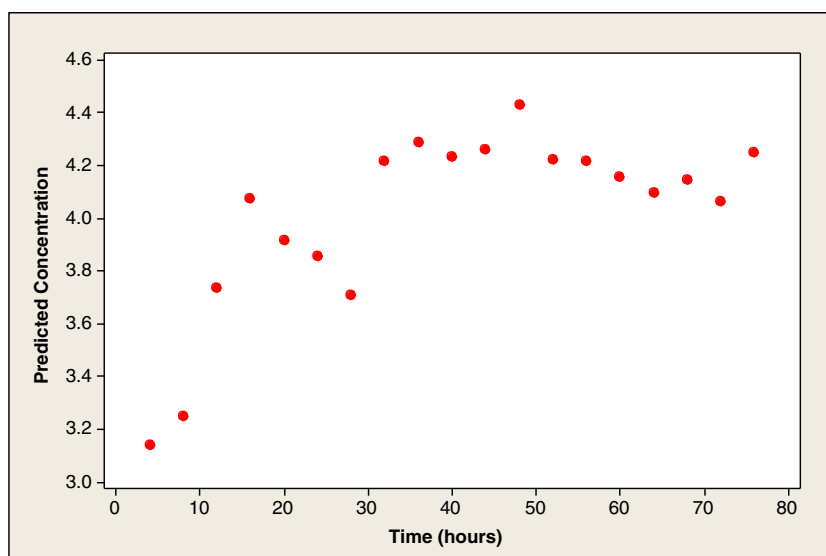
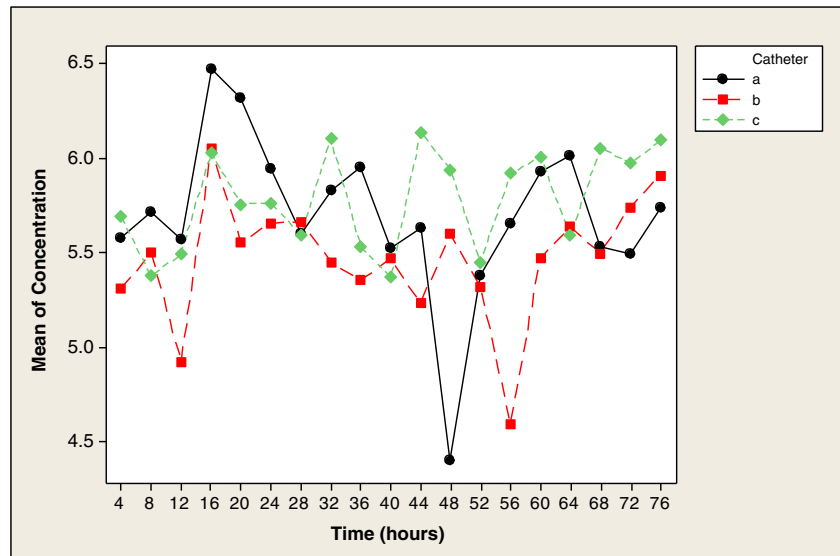


Fig. 3 Mean concentration averaged over 6 patients versus time, by catheter, $\text{TNF}\alpha$



$\text{FGF}\beta$ concentration which may explain these border line F-statistics for the non-linear effects of time, which may be oscillations about the trend line of biological significance.

Discussion

Understanding the vascular territories of the skin, angiosomes [3, 10] and perforasomes [2], and the concept of safely capturing several vascular territories in flap design with detailed knowledge of the vascular anatomy, has improved the design and safety of flap transfer. The interconnections between

vascular territories, which can include ‘direct’ or ‘true anastomoses’ being vessels that do not change in calibre, and reduced calibre ‘indirect’ vessels or ‘choke vessels’ [2], relates to the number of angiosomes that can be reliably used, and the concept of the ‘functional angiosome’ [1]. However, the mechanisms of choke vessel or indirect vessel opening has yet to be elucidated, and this knowledge may lead to the ability to influence vessel opening, and increase the functional angiosome size. This may be of great value in certain scenarios of flap transfer or impending partial necrosis, and in other forms of necrosis caused by insufficient blood supply for example meningococcal septicaemia [1].

Fig. 4 Predicted concentration by time, $\text{TNF}\alpha$

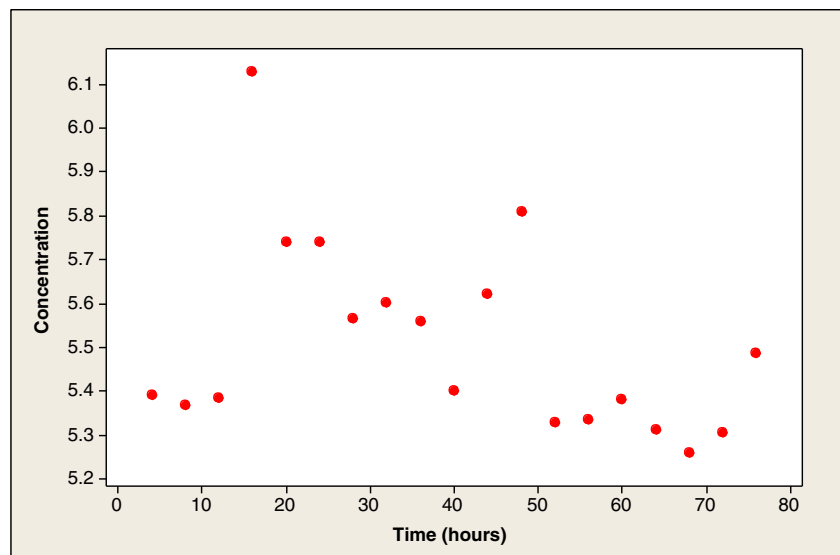
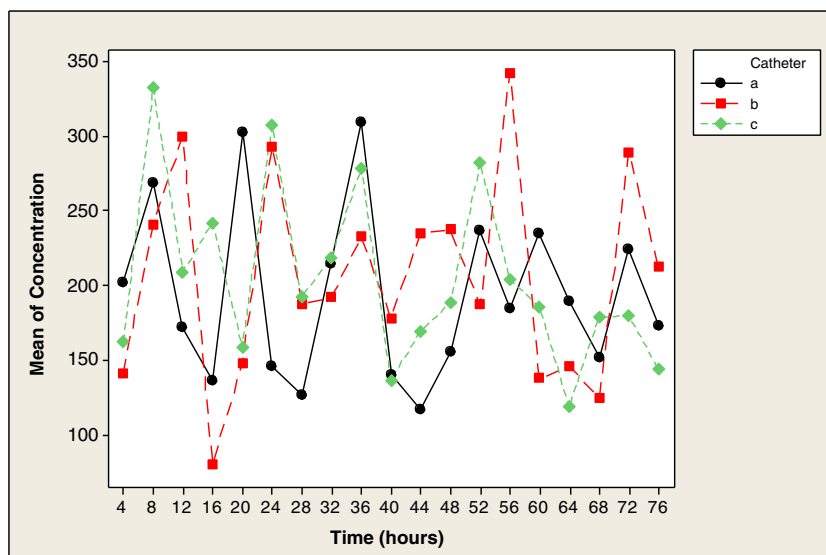


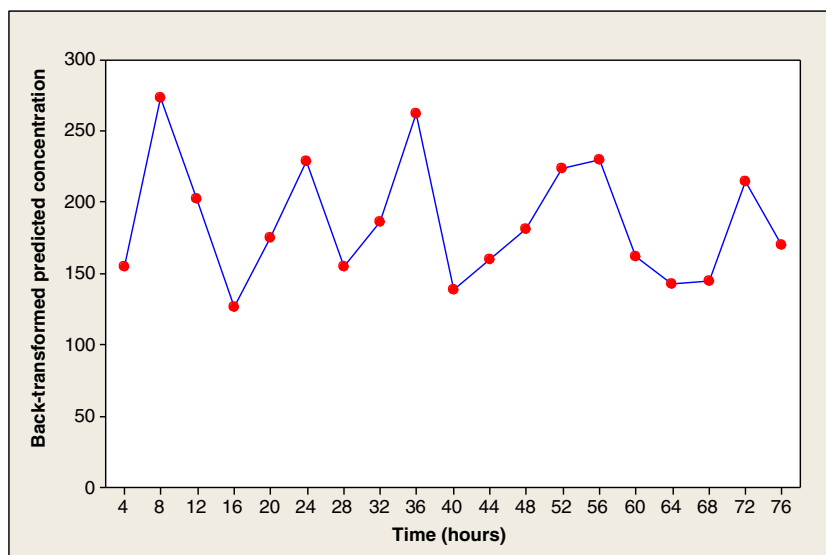
Fig. 5 Mean concentration averaged over 5 patients, versus time, by catheter, FGF β



Delay has been used as a mechanism for improving flap survival and experimentally to improve understanding of the chemical signalling that may be involved in the dilation of choke vessels and intraflap vessels. Many animal studies have been designed looking at the chronological sequence that may occur in choke vessel opening [4, 11–13]. They concluded similarly that surgical delay results in dilatation of the existing vessels with maximal effect in the zone of the choke vessels, rather than improved blood flow being due to neovascularisation or angiogenesis. The timing of improved blood flow across angiosomes was maximal at 48–72 h [4, 13], with a

marked increase in diameter in vessels in the flap particularly in the zone of the choke vessels [4, 11, 13]. A detailed study by Dhar and Taylor in 1999 [13] involved 200 rabbits and 17 dog models to look at choke arterioles in the early and late post delay period. They summarised that events due to delay occurred in four phases, phase 1 representing the initial vasoconstriction and then vasodilation up to 24 h, with phase two occurring between 24 and 72 h, especially between 48 and 72 h with an accelerated increase in vessel diameter especially at the choke vessel level with cell division in the vessel wall. Phase 3 occurred between 72 h and 7 days with more gradual

Fig. 6 Predicted back-transformed (squared) concentration by time, FGF β



dilation, and phase 4 from 7 days to 1 year with permanent dilation of the choke vessels. In planning our clinical study, we therefore decided to focus on the first 4 days following DIEP flap transfer to investigate cytokines that may relate to this process.

Mechanisms of active dilation of the choke vessels proposed include physical changes such as the pressure, ‘stretch’ and pulsatile effect of the blood flow [13], reactive hyperaemia (and hyperadrenergic) states in the proximal zones [14], and chemical mechanisms where ischaemia may act as the primary stimulus, and although known to stimulate neovascularisation [13] (via hypoxia inducible factors HIF [15, 16]) hypoxia has a less clear effect on choke vessels. Pharmacological agents that have experimentally increased flap survival in animals include guanethidine [17], topical nitroglycerine [17, 18], prostaglandin E1 and E2 [17, 19, 20], FGFβ [21], platelet-derived growth factor (PDGF) [22] and thromboxane A2 synthase inhibition [23]. Decreases in survival have been shown with thromboxane, prostaglandin F1α [23] and epinephrine [24]. And finally, there is still the question of why choke vessel dilation becomes permanent.

In this novel clinical study using microdialysis catheters, we chose three cytokines previously shown in animal studies to upregulate in flaps [25–29]. IL-6 and tumour necrosis factor alpha (TNFα) are both involved in signalling the acute phase response and fever, and are inflammatory cytokines, and play key roles in the cross-talk between cytokines. IL-6 is an important pro-inflammatory mediator that can promote the release of TNFα by activating leukocytes, and TNFα is one of the most important pro-inflammatory cytokines. FGFβ is part of a family of growth factors involved in wound healing, embryonic development and is one of the most powerful angiogenic factors identified.

Many animal studies of flaps have been performed to look at dermal cytokine expression with an aim of understanding flap ischaemia. In 1995, Most et al. [26] performed a rat study using dorsal flaps with biopsies taken at 0, 8, 16, 24 and 48 h after the flaps were raised and at varying distances from the end of the flap (0.5 cm to 10 cm). Cytokine expression was examined with the highest detected at 8 h post-operatively for PDGF, transforming growth factor beta (TGFβ) and FGFβ, and each of these cytokines had a 20-fold increase ($p < 0.002$). IL-6 expression was also highest at 8 h with a 14-fold increase. Authors noted that there was a dramatic and

early cytokine expression at flap bases compared with their ischaemic tips. Our study similarly did not find increased cytokines in zone 4 furthest from the pedicle, although there was no significant difference in cytokine concentration in any of the zones.

In a delayed TRAM flap model in rats published by Wong et al. in 2004 [27], FGFβ was measured by biopsy and enzyme-linked immunosorbent assays. A surgical delay was performed 7 days ($n = 12$), 14 days ($n = 10$) or 21 days ($n = 7$) before the flap was raised. FGFβ was found to be significantly higher in all delayed flaps than the control flaps with no delay ($n = 6$) at day 3 post-operatively. There was no difference in FGFβ across zones 1–4. The authors concluded that the increase in FGFβ at day 3 in delayed flaps may be a factor in improved flap viability, and that further investigation was required. Lineaweaver et al. demonstrated a significant increase in cytokine expression of FGFβ and vascular endothelial growth factor (VEGF) in delayed rat TRAM flaps post-operatively, again with no significant differences between zones [28].

Cytokine expression is also increased following ischaemia-reperfusion. A rat study by Zhang et al. in 2005 subjected rat gracilis muscle to two episodes of ischaemia, a primary episode for 1 h and then a 24 h later a secondary ischemia of 4 h [29]. Gene expression of TNFα, interleukin-1 beta (IL-1β) and PDGF mRNA was determined by PCR at 4 and 18 h after the primary ischaemia and at 0, 4 and 18 h after the secondary ischaemia. IL-1 was upregulated 4 h after the primary ischaemia and PDGF was upregulated immediately after the secondary ischaemia. TNFα was significantly upregulated 18 h after secondary ischaemia. Our highest concentration of TNFα was at 16 h, and this was significantly higher than the concentration at 12 h post-operatively ($p = 0.002$).

A more recent animal studies by Mao in 2019 used biopsies in choke vessel zones of mice flaps to compare single and multiple perforator based flaps [30]. Blood flow in the choke zones was measured by laser Doppler flowmetry at 6 h, 1, 3, 5 and 7 days. Histological analysis showed morphological changes including increase tortuosity, dilation and thickened walls of choke vessels, with expansion into the choke zone by day 3. Endothelial nitric oxide synthase (eNOS), metalloproteinase 2 (MMP-2), hypoxia-inducible factor 1α (HIF-1α) and intercellular adhesion molecule 2 (ICAM-2) were measured at these

time points. Endothelial NOS increased expression at 6 h, endothelium being the first site stimulated by shear stress. MMP-2, secreted by macrophages, is associated with vascular remodelling and had significantly increased by days 1 and 3 and HIF-1 α and ICAM-2 increased by day 3. Luo et al. in 2019 similarly investigated the haemodynamic and morphological changes in choke vessels in a new rat flap model, investigating the role of known angiogenic factors HIF-1 α , iNOS and VEGF in the choke zone [16]. Histologically, choke vessels were open at day 3 and microvascular density increased until day 5, with iNOS significantly increased on day 1 (consistent with massive opening of choke vessels) before gradually decreasing, and HIF-1 α expression reached a maximum at day 5 post-operatively. Luo et al. demonstrated previous experimental knowledge that HIF-1 α expression may be involved in the early vascular remodelling process by inducing iNOS and VEGF expression. Morphological elements of both these animal studies demonstrate choke vessels opening in first few days as originally proposed by Taylor et al. [3, 13], and also provide further insight as known angiogenic factors are present in the choke vessel zones at a time when the choke vessels are becoming true anastomosis. Luo et al. also make the point that there are no previous animal studies with single pedicle multi-territory perforator models until theirs, a model that would be comparable with clinical DIEP flaps again highlighting the need for more animal but also clinical studies for further understanding of the processes of choke vessel dilation permanence and partial necrosis [16].

Manipulation of flap survival has been attempted in many animal studies, largely based on the knowledge of theoretical negative impact of pro-inflammatory cytokines, such as TNF alpha and IL-6 that we have investigated, and pro-angiogenic factors such as FGF β and VEGF [25, 31, 32]. TGF β , fibroblast growth factor, endothelial growth factor and VEGF have shown some of the more impressive results in flap survival [21, 32–43]. Many of the outcome markers examine the percentage of flap necrosis at 1 week post-operatively, reflecting a number of processes including the opening of choke vessels in the initial few days and angiogenesis contributing thereafter, and any effects ischaemia-reperfusion injury during free flap transfer. In addition, application of growth factors to flaps range from the time of raising the flap to as much as 10 days pre-operatively, further complicating

extrapolation of timing in relation to choke vessels. Similarly, targeting the negative effects of inflammatory factors, Entercept, a soluble TNF α binding protein, has been shown to offer protection against ischaemia reperfusion injury in a rat transverse rectus abdominus myocutaneous (TRAM) flap model [44]. Use of growth factors have also reduced the expression of inflammatory mediators [45]. Other studies demonstrate success with hirudin [25, 46] by improving microcirculation, but also by reducing inflammation. These studies present further possibilities for increasing flap survival.

Clinically, varying scenarios would need to be encountered to appreciate patterns of cytokine release in relation to outcome, including partial necrosis. There may be differences across zones and with time, and also between different flap compositions [47]. The use of microdialysis catheters is a more acceptable method of investigation of cytokine levels than biopsies as used in animal studies. Microdialysis fluid can be collected continuously and high cut-off catheters would appear to collect cytokines IL-6, FGF β and TNF α all less than 100 kDa. It would be useful to directly compare the levels of cytokines in biopsy specimens with microdialysis catheters. Whilst it may seem desirable to have a non-flap catheter to measure systemic response to surgery and catheter insertion, the investigation of choke vessels is an intra-flap phenomenon (with zone 1 as the baseline). The use of high cut-off catheters as a research tool in plastic surgery is not reported in the literature, and without further study it is difficult to ascertain whether there could be confounding factors in the measurement of molecules observed in the biological setting. For example, could circulating molecules close to the membrane pore size cause temporary occlusions altering the amount of researched molecule reaching the microvial? We chose a low flow rate through the microdialysis pumps to maximise the concentration of cytokine collected, necessitating a minimum of 4 hourly intervals which is still more frequent than 12 h biopsy intervals in many animal studies.

Conclusions

Microdialysis using high-cut off membrane catheters, allowing the sampling of larger molecules such as cytokines, has been used to investigate the cytokine changes

that occur across multiple angiosomes following flap surgery. Further research and a better understanding of this initial time period when choke vessel opening occurs may ultimately allow manipulations to avoid partial necrosis and improve the vascular territory.

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Authors contributions CJ Tollan and IR MacKay contributed to the study conception, and all authors to the design. Material preparation and data collection were performed by CJ Tollan and IR MacKay. Analysis was performed by N MacFarlane and CJ Tollan. The first draft was written by CJ Tollan and all authors commented on a previous version of the manuscript. All authors read and approved the final manuscript.

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Data availability Not applicable.

Compliance with ethical standards

Conflict of interest C. J. Tollan, Niall G. MacFarlane, and Iain R. MacKay declare that they have no conflict of interest.

Ethical approval Local institutional Ethics Committee approval was granted by Glasgow Royal Infirmary Research and Ethics Committee (REC reference number: 08/S0704/20) and certify that the study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki.

Patient consent Patient participants in the study had informed consent as approved by the ethics committee (REC reference number: 08/S0704/20) and signed a consent form (including the patients' ability to withdraw from the study at any time without detriment to care).

Informed consent The patient information sheet as approved by the Research and Ethics Committee (REC reference number: 08/S0704/20) as above for the purpose of consenting the patient explains that their data, anonymised, as the results of the study would be published in the medical literature.

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